I. *"Nitragin" and the Nodules of Leguminous Plants.*

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Communicated by Professor H. Marshall Ward, *F.R.S.*

[Plate 1.]

The investigations, which form the subject of this paper, were begun by me in the autumn of 1897, at the suggestion of Professor Marshall Ward.

My chief aim was to study the nature and properties of the so-called "nitragin," a patent germ-fertiliser, recently introduced by Drs. Nobbe and Hiltner for use in agriculture.

Up to this time the results obtained with it have been of a more or less conflicting nature ; for instance, Mr. Howard Ryland (37) reports that with green peas, broad beans, and sweet peas, he has obtained favourable results, but that the effect produced upon French beans was very doubtful. Messrs. Sutton and Sons (37) have found beneficial results to accrue from the use of nitragin, chiefly, however, as regards root growth. These investigators are, I believe, repeating their experiments this year, the results of which are not yet published.

In "Nature," 1897, are recorded some experiments carried out by Professor Somerville, in the garden of the Durham College of Science. On ordinary garden soil, he grew crops of peas, beans, Lucerne, and red clover. The plots chosen had been previously employed for the cultivation of peas and beans, but had never before been planted with Lucerne or clover. It seemed probable, therefore, that the conditions were especially favourable for the advantageous use of nitragin in these two cases. Nevertheless, an increase in the yield was only obtained in the case of peas, "and even then the variations in the weights of produce were too small to make it possible to say definitely that the inoculating substance affected growth either one way or another."

Such indecisive results as these pointed to the necessity for a definite study of the question, with a view to criticising the value of nitragin to the agriculturist.

This naturally involves the study of the life-history of the organisms concerned in the formation of the nodules, as regards their behaviour both within and without the plant. Before describing the methods which I have adopted, I propose to give a brief account of the work already done in this direction.

An account of this work very naturally falls under two heads, viz. : i. Investigations of the morphological characters of the tubercles and their contents; ii. The study of their physiological importance in the life of the Leguminous plant with which they are connected.

As early as 1867–78, tubercles upon the roots of many plants had been observed and carefully examined by ERIKSSON and WORONIN (1); the former, in particular, devoting his attention to those occurring in the Leguminosæ, which he attributed to the action of a true fungus.

Shortly after his work, appeared that of KNY (2) and PRILLIEUX (3), each of whom regarded the filaments within the cells of the tubercles as a type of plasmodium, such as occurs in the Myxomycetes. In 1885, BRUNCHORST (4) refused to accept the parasitic nature of the " Bacteroids," and suggested that they might be some kind of ferment secreted by the protoplasm.

A long and detailed account of the histological characters of the tubercles of *Lupinus* and *Robinia* was published in 1887 by TSCHIRCH (5). He considers the tubercles developed in these two genera to be typical of the two classes found in all the Leguminosæ, and describes in detail their respective relations to the vascular bundles of the roots, and the special characters of the inner " Bacteroid " tissue, noting in particular the absence of filaments along with Bacteroids in the cells of the tubercles of *Lupinus*. In accordance with BRUNCHORST, he denied the parasitic nature of the Bacteroids; indeed, he considered the tubercles themselves to be merely storehouses of reserve material, chiefly albuminoids and possibly also starch.

In the same year MARSHALL WARD (6) first clearly demonstrated the parasitic nature of the filaments present in the cells of the tubercles of *Vicia Faba*. He described and figured the entrance and passage down the root hair of the " infection tube," also its growth across the cells of the cortex, into the inner tissues of the root. At the point on a root hair where infection had taken place he noted a bright " spot of infection," and also a bladder-like swelling of the wall. This latter phenomenon he explained as due to unequal growth on the two sides of the hair after the piercing of the wall by the parasitic organism. He described in addition the trumpet like expansions of the filaments at points where they cross the cell-walls, and the frequent spherical or pear-shaped swellings which occur upon them within the cells. From these swellings he saw the budding-off of minute gemmules, which by repeated multiplication within the cells gave rise to the dense mass of Bacteroids with which they come to be filled.

These observations were, in the main, confirmed by PRAZMOWSKI (7) in 1888, and again by MARSHALL WARD (6) in 1889. These authors differed widely, however, upon the systematic position of the parasite; the latter suggested the possibility of classing it with the Ustilagineæ, the former believed it to be a true Schizomycete, enclosed in hypha-like strands of a plasmatic substance.

In 1888 BEYERINCK (8) explained the filaments (*Schleimfäden*) as remains of the nuclear substance, which after cell division have not returned to the cytoplasm or the nucleus. The Bacteroids he regards as the infecting organisms, and having succeeded in cultivating, on gelatine media, micro-organisms which were capable of producing direct infection of Leguminous plants, he concluded that these were true Bacteria, which were, however, specially adapted to a parasitic mode of life within the Leguminosæ.

In 1894, however, BEYERINCK changed his views upon the nature of the *Schleimfäden*. At this later date he regarded them as representing the cell-walls of Bacteria, some of which remain embedded in the mucilage, while others are completely forced out during the formation of the filament. By means of this covering the Bacteria within the filaments are protected from the action of the cell-plasm, which, along with the nucleus, the author considers has some mechanical action over all the filaments. He still describes the filaments as connecting the nuclei of adjacent cells.

VUILLEMIN (9), on the other hand, propounded an exactly opposite theory to the above—the filaments he regarded as the hyphæ of a true fungus, in which he claims to have seen spore formation, and which he groups with the Chytrideæ, under the name *Cladochytrium tuberculorum*—but the Bacteroids he believed to be of proto-plasmic origin, and formed by the breaking up of a network of protoplasm. This appearance of a network VUILLEMIN obtained by leaving sections of tubercles of *Vicia hirsuta* in water for about four days. It is obvious that such treatment would necessarily cause considerable changes to take place in the contents of the cells, consequently any observations based upon such a method can scarcely be relied upon.

A similar origin for the Bacteroids was suggested by FRANK (10) in 1890. He, however, was of opinion that the network consisted of an intimate mixture of phanerogamic and fungal protoplasm, which he terms " Mykoplasm."

From characters possessed in common by this mykoplasm and the infection tubes and filaments, FRANK concluded that they are of like origin, and are, in fact, formed by the plant itself as a means by which it conducts to the inner cells the symbiotic organisms (minute cocci) which are destined to grow there. In this way he explained the absence of filaments in *Lupinus* and *Phaseolus*, for since in these genera infection took place directly through the cells of the piliferous layer, tubes and filaments were no longer necessary, and consequently, being organs of the plant itself, they could be, and were, dispensed with. Throughout his work FRANK insisted that the Bacteroids are not themselves the germs, but that they contain them, and that, after the decay and death of the tubercles, the main substance of the Bacteroids is absorbed by the plant, whilst the contained cocci are returned to the soil.

In support of this theory FRANK adduced the facts that, though he had found Bacteroids in aërial organs of plants bearing tubercles, he utterly failed to detect them in plants devoid of tubercles, and that *Phaseolus*, a genus in which he found Bacteroids even in the cotyledons, was capable of producing tubercles even in sterilised soils. It is very difficult to understand how such results have led FRANK to this opinion, for they appear clearly to point to the conclusion that the Bacteroids *are* themselves the germs, whatever their contents may be. In this connection, too, FRANK reports the occurrence of Bacteroids in the parenchymatous tissues of aërial organs of lupine, pea, and bean, and in the cotyledons of the bean. I may at once state that, in accordance with SCHNEIDER and others, I have utterly failed to confirm these observations. In no case were Bacteroids visible in the cells of any other organs than the tubercles themselves. In addition, although I have made repeated attempts, using the methods which FRANK suggests, to determine the nature of the contents of the Bacteroids, I have been quite unable, even with high power objectives, to detect any structure which could justify the conclusion which he has drawn, viz., that they contain minute cocci, the true germs.

In 1879 FRANK had named the tubercle organism *Schinzia leguminosarum*, that is, he considered the filaments to be true fungal hyphæ. At this later stage of his work, however, he has rejected this old name, and following BEYERINCK's view, has classified it with the true *Schizomycetes*, under the name *Rhizobium leguminosarum*. His histological results consist practically of a confirmation of those already published by MARSHALL WARD. He, however, made some additional observations on the root hair tubes, which are of considerable interest. He studied these tubes before they had grown all the way down the hair. He was thus able to note the character of the growing end, and called special attention to its open appearance, which seemed to merge gradually into the contents of the hair. Such a growing apex was obviously totally unlike that of a fungal hypha, and argued strongly against the hyphal nature of this tube.

In 1891, FRANK's results with *Phaseolus* and *Lupinus* were contradicted by LAURENT (12). In both these genera he described and figured filaments in the cells of very young tubercles, though he admits that they were not to be seen in older specimens.

As regards its systematic position, LAURENT classed the organism with *Pasteuria ramosa*, described by METCHNIKOFF, in a group intermediate between the *Schizomycetes* and the lower fungi.

In 1893, FRANK's views were again attacked by SCHNEIDER (13), who denied the presence of bacteroids in tissues other than the root, and also denied the presence of air passages around each of the tubercle cells. This latter result had been published by FRANK (10) in 1892, in a paper upon the evolution of gas from Leguminous nodules.

During the year 1898 ZUKAL (14) hinted that the *Rhizobium leguminosarum* may

be more correctly included amongst the Myxobacteriaceæ ; LAFAR (25), however, in his recently published "Technical Mycology," has accepted the view that the Bacteroids are involution forms of a true Bacterium, which enters the root-hair in the form of a filamentous zoogloea.

In 1887, TSCHIRCH (5) denied the parasitic nature of the Bacteroids, on the grounds that he had failed to cultivate them outside the plant, or to extract from Leguminous soils any Bacterium corresponding in form to the Bacteroids.

However, in 1888, BEYERINCK (8) overcame this difficulty by the use of gelatine media, containing suitable proportions of an extract of pea stems or leaves, asparagin, and in some cases sugar. By this means he successfully isolated from the tubercles a micro-organism, which he named *Bacillus radicicola*, and with which he was able to secure artificial infection in *Vicia Faba*.

BEYERINCK'S opinion was that this species is common to all Leguminosæ, and that its special characters in individual cases must be attributed to the influence of the particular host plant rather than to any specific difference in the organism itself.

In 1894, SCHNEIDER (13) made a study of the organisms present in different genera and species, and described how they varied in form, colour, &c. ; these variations in form have been figured by MÖRCK (1891).

In January of 1897, MAZÉ (17) published results of some experiments to test the power of these organisms to absorb atmospheric nitrogen, independently of the plant. For this purpose he grew cultures on both solid and liquid media, and in each case he claims to have obtained a very vigorous growth of the organism, which he attributes chiefly to the aëration of the culture flasks, by a current of air, drawn through the apparatus by an aspirator. The numbers, which he quotes, show a relatively large absorption of nitrogen during the experiment—but since the difficulties of manipulation and of analysis are obviously very great, such results must only be received with great reservation. The increase in nitrogen, in each case, only amounts to a few milligrams, a quantity scarcely beyond the range of error of even the most skilled gas analysts.

Also during 1897 ZINSSER (18) working in Leipzig upon the conditions of infection with Rhizobium made use of silicic acid jelly, which was introduced by WINO-GRADSKY for the isolation of nitrifying bacteria. This medium ZINSSER has found more successful than the ordinary gelatine employed by BEYERINCK and others. Amongst the conclusions which he drew from his work are the following :—

1. The Leguminosæ are not hereditarily infected with Rhizobium.
2. Bacteroids do not occur in aërial organs, or in the inner tissues of roots.
3. Other bacteria, cultivated from aërial organs, are incapable of producing tubercles.
4. The tubercle organism is unable to wander upwards or downwards in the plant tissues.
5. Provided the plant is healthy, and is supplied with all the conditions necessary to its healthy development, the age of the plant and the time of the year has no effect upon the infecting power of the Rhizobium.

6. Under the conditions which he supplied, the organisms were unable to live when the air was deprived of all combined nitrogen.

He does not, however, seem to have arrived at any definite conclusion upon their power to assimilate free nitrogen, independently of the Leguminous plant.

The study of the *physiological* importance of a supply of nitrogenous food to our crops dates from the well-known researches carried out by LAWES, GILBERT, and PUGH (19) at Rothamsted in 1861. These researches were followed by WARINGTON'S (20) work on nitrification in soils, and by the repeated investigations of HELLRIEGEL and WILFARTH (38), and of FRANK (10) into the phenomenon of the assimilation of free nitrogen in the vegetable kingdom. In 1890, working in conjunction with OTTO, FRANK (11) came to the conclusion that free nitrogen assimilation is not confined to the Leguminosæ, but is comparatively common amongst plants, *e.g.*, it occurs in the fungi, in algæ and mosses, and in such phanerogams as *Avena, Brassica, Sinapis*, &c. These authors asserted in addition, that Leguminous plants absorb nitrogen through their leaves, without the aid of Bacteria. This opinion was refuted in 1892 by KOSSOWITSCH (21), whose experiments led to the conclusion that the free nitrogen was absorbed through the roots and not through the aërial organs.

Since FRANK'S work upon non-Leguminous plants was carried on in unsterilised soils, his results cannot be considered trustworthy, and they were in fact, contradicted later by LOTSY (39), SCHLOESING and LAURENT (12) and others. LAURENT in 1892 showed conclusively that Leguminous plants were able to make use of atmospheric nitrogen, and moreover that this power was closely connected with the presence in the soil of certain microbes, concerned in the formation of the tubercles on their roots. In 1889 VINES (22) had shown that the formation of the tubercles was favoured by a diminished supply of nitrates to the culture soils, and in 1893 NOBBE and HILTNER (23) came to the conclusion that those tubercles, in which the organism does not reach the bacteroid stage, are injurious rather than useful to the plant, since unchanged bacteria present in the tubercles seem to have no connection with the nitrogen fixed by the plant.

In 1894, MARSHALL WARD (6) called attention to the four main views concerning the means by which Leguminous plants make use of free nitrogen. They are briefly as follows :—

    i. That gaseous nitrogen is fixed directly by the plant.

    ii. That nitrogen is fixed by bacteria, and the resulting compounds passed on to the plant.

    iii. That nitrogen is fixed by the Leguminous plant, owing to power obtained through the stimulating action of the root-nodules.

    iv. That the root-organisms are the nitrogen collectors, and the Leguminous plants absorb these together with their accumulated stores.

Which of these views is the most tenable is still an open question, though the

character of the tubercles at the end of the vegetative period seems to lend support to the last rather than to any other.

As has been already mentioned, in 1889, MARSHALL WARD proved the possibility of the production of tubercles by direct infection from without. His method was to use slices of dried tubercles, applied to seedlings grown in culture solutions. Later investigators have employed sterile soils variously inoculated by means of extracts of nodules of different plants, or of soil in which Leguminous plants had been previously grown. By such means NOBBE and HILTNER (23) have tested the action of the organism from one genus upon an individual of some other genus, and have drawn the conclusion that, whilst each species is with most certainty infected by the organism peculiar to itself, yet, within the limits of the various tribes of the order, it is possible to produce infection by the use of the organism proper to one species upon seeds of some other species with a tolerable degree of certainty.

Experiments in which the species in question belong to different tribes usually give negative results. From the descriptions given by these authors it is somewhat difficult to realise whether they have used extracts of soils or tubercles as the inoculating material, or whether they made intermediate pure cultures of the organisms outside the plant. Unless this latter method has been adopted their results can scarcely be trusted, since it is obviously impossible to guarantee the purity of the organism supplied to the soils in which the various species were grown.

Some interesting results, published by FRANK (10) in 1888 and 1890, upon the effect of the composition and sterilisation of the soil upon tubercle formation should also be noted. He showed that in unsterilised soils the formation of tubercles depends chiefly upon the nature of the soil employed, and came to the conclusion that the most healthy development of a Leguminous plant is obtained in a sterilised soil provided it be rich in nitrates, but that if the soil be devoid of nitrates the best results are obtained when no sterilisation has taken place.

In October, 1897, I began the study of this subject by the microscopic examination of nodules of different ages from the roots of different genera and species of the Leguminosæ, amongst which were *Vicia hirsuta*, *V. Faba*, *V. sativa*, *Pisum sativum*, *Hippocrepis multisiliquosa*, *Trifolium maritimum*, *Lathyrus aphaca*, *L. Chymenium*, *Lupinus albus*, *L. luteus*, *Phaseolus multiflorus*.

It was at once obvious that the characters exhibited by the nodules of *Lupinus* and *Phaseolus* were in many respects distinct from those of the other genera, consequently, in order to definitely limit my work, I determined to confine it almost entirely to the study of the species *Vicia hirsuta* and *Pisum sativum*.

In each case tubercles about the size of a pin's head are visible within fourteen days of sowing, and in about three weeks they are present in considerable numbers on both main and lateral roots.

Those of *Vicia* and the majority of the larger ones of *Pisum*, as a rule, branch in a palmate manner, and sections show that the vascular bundle system branches through

them in the same way. The distribution of the vascular bundles has been already fully described by VUILLEMIN (9) in *Vicia hirsuta* and need not be further discussed.

For the preparation of preserved material I tried various methods of hardening, such as a saturated solution of mercuric chloride, Hermann's solution, Flemming's solution, absolute alcohol, and boiling in water for a few minutes, and then transferring gradually to absolute alcohol. I obtained the most satisfactory results with Flemming's more concentrated solution and with ordinary absolute alcohol. For some time I made use of both hand sections and microtome sections of paraffin material. The latter method I afterwards abandoned however, since I found the tubercle tissues very difficult objects to stain upon the slide, and also ordinarily thin hand sections serve better for the examination of the filaments within the cells—a point to which I wished to devote special attention.

It seems unnecessary to enter into details of the ordinary anatomical characters of these nodules, since all the main points have been already described and figured by MARSHALL WARD, FRANK, and others, and although these observers differ so greatly from one another from a theoretical standpoint, their descriptions of the morphological characters are practically identical.

By examination of the hairs of roots, on which the tubercles were first visible, I obtained abundant evidence of the parasitic nature of the organism. The infection tubes are made very easily visible if the root be first treated with Eau de Javelle or potash. In all cases, whether at the tip or on the side of a hair, a bright spot of infection was obvious, and also a bladder-like swelling of the hair at this point of attack. I examined the infection tube at all stages of its growth down the hair until it reached the cortical cells of the root, when sections become necessary in order to trace its growth further. For this purpose simple hand sections of fresh material, treated with Eau de Javelle or potash, give excellent results. By this means the course of the tube across the cortex, and its branching into all the cells of the tubercle, can be seen clearly. A more detailed examination of the sections shows the trumpet-like swellings described by MARSHALL WARD, where the tubes cross the cell-walls, also the numerous spherical or pear-shaped swellings formed on the tubes within the cells. In many cells breaks may be seen in the tubes—each portion ends abruptly in a fine point—the points being directed towards each other. (Plate 1, figs. 1–4.)

It seemed at first possible that excessive swelling by Eau de Javelle might have caused this apparent bursting of the filaments, but the study of sections mounted in pure water and of hardened material showed the phenomenon to be quite normal. In sections of older tubercles the thicker filaments crossing the cortex are no longer to be seen, but those in the main tissue of the tubercles persist until decay has set in.

Having thus far confirmed the work of previous observers, my next aim was to obtain some re-agent which would stain these filaments without staining the Bacteroids. With such stains as iodine, gentian violet, and hæmatoxylin, I could only secure very

inadequate results.   I next tried a solution of gold chloride (·5 per cent.).   Since this stain must be used upon fresh material, I treated the tubercles *en masse* for microtome work, leaving them in the stain from one to twenty-four hours.   Hand sections were only left thus for from ten to fifteen minutes.   In either case the material was then quickly washed with water, and transferred to a solution of formic acid (·25 per cent.) in the dark for twenty-four hours, or for the same time to water acidulated with acetic acid, in the light.   The sections were then washed well in water and placed in formic glycerine, or if intended for embedding, the material was transferred gradually to absolute alcohol and thence to paraffin.*   With this method I obtained very promising results, the contents of the filaments were stained deeply, whilst the limiting layer remained colourless.   Some sections gave results which suggested that the contents were broken into short rodlets, but the stain was not sufficiently differentiating to admit of any definite conclusion upon this point.

These hints of the nature of the filaments were, however, quite satisfactorily supplemented by the use of a method given by STRASBURGER (24) for differentiating fungus hyphæ in the tissues of the host.   The method† is as follows :—Sections hardened in alcohol (best without previous treatment with chromic or osmic acid) are placed for about two hours in alcoholic potash (1 part 5 per cent. potash to 3 parts absolute alcohol) and then passed into Eau de Javelle for ten minutes.   From this solution they are transferred to the dye, which is prepared by mixing an alcoholic solution of aniline blue with orseillin, drop by drop, until a violet solution is obtained. This mixture is acidulated with a few drops of glacial acetic acid.   The sections remain in the stain for two hours, and are then transferred directly to dilute glycerine, and finally mounted in glycerine.

By this method, even with a $\frac{1}{6}$ objective, the structure of the filaments was clearly seen, and examination with ZEISS' $\frac{1}{12}$ Hom. Imm. put beyond doubt the accuracy of the view already suggested by the sections stained with gold chloride.   A reference to fig. 9 will show that the filaments consist of numbers of straight rodlets, lying with their longer axes parallel to the direction of growth of the filament.   Where the swellings occur on the filaments they are present in larger numbers, and finally, as further sections showed, the tube bursts and the rodlets are liberated into the cell cavity (fig. 10).

By cutting transverse sections of tubercles, I succeeded in getting a view of the filament crossing the cell wall : the figure obtained resembles closely an ordinary sieve plate (see fig. 11), and as far as could be determined the rodlets actually pierce the wall, absorbing the middle lamella only, not the outer layers.

By staining with methyl violet and fuchsine, a method described by LAFAR in his

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* I also tried the use of gold chloride upon preserved material—in this method the sections are treated with fresh lemon juice after staining with the gold.   In no case, however, was any coloration produced.

† The directions given by STRASBURGER are quite vague as regards strengths of solutions and the length of time for each process.   The details given above are those from which I obtained my best results.

" Technical Mycology" (vol. 1, pp. 340–351), I obtained further confirmation of the above results, though the differentiation with aniline blue and orseillin was the more successful of the two.

BEYERINCK (8), in a paper published in the 'Botanische Zeitung,' 1888, has represented a definite relation as existing between these filaments and the nucleus of the cells. He figures them invariably crossing the nucleus, or at least in intimate contact with it. This relation between the filaments and nuclei was still maintained by him in 1894, though he had then abandoned his theory of the nuclear origin of the filaments. In my sections, however, I have not found this constant relation between the nucleus and the filaments. In some cells, it is true, they are seen in close contact with the nucleus, but in others they appear to have taken a course across the cell quite independent of the position of the nucleus.

I may here note briefly that, in accordance with FRANK's descriptions, I have thus far failed to detect any trace of filaments in the tubercles of *Lupinus* or *Phaseolus*, and though I have sectioned tubercles of very various ages, I can find nothing corresponding to the figures published by LAURENT in 1891.

Having regard to the very different theories which have been propounded to explain the nature of the organism in these tubercles, it is not remarkable that a corresponding variety of opinion has arisen as to the presence and constitution of a membrane bounding the infection tube and its branches within the cells of the tubercle.

TSCHIRCH (5) in 1887 asserted that the tubes possessed no membrane, and that with iodine and H_2So_4 they were coloured yellow. BRUNCHORST in 1885, and FRANK in 1879, had described a definite bounding layer; but in 1887 and 1889, FRANK changed his opinion and described the filaments simply as denser, more resistant strands of plasmatic substance or " Mykoplasm."

In 1887, MARSHALL WARD (16) detected a clearly defined membrane to the infection tube and to the tubes in the cells of the cortex, but did not observe any such layer within the inner cells. These results were confirmed in the following year by PICHI and VUILLEMIN, both of whom claimed to have obtained the reactions of true cellulose. KOCH (26) in 1890 supported these observers, and described his reactions as follows : sections of alcoholic material are treated with Eau de Javelle for a few hours, washed in water, and then transferred to a solution of chlor-zinc iodine (40–60 per cent.). By such a method he claimed to prove beyond doubt the existence of a cellulose membrane in *Pisum sativum, Vicia Faba, Trifolium pratense, Onobrychis sativa*, &c.

Throughout my study of these tubes, the results which I have obtained have led to doubts of the presence of cellulose in their membrane. Both with gold chloride and aniline blue and orseillin a clear unstained matrix could be seen, in which the rodlets were embedded. All my attempts to detect cellulose by KOCH's method, or with iodine and sulphuric acid, utterly failed; as TSCHIRCH has described, the contents of the tube stained yellow, but the matrix remained completely colourless. On the

contrary, the cell walls of the surrounding tissue and the root hairs gave the usual cellulose reaction quite successfully.

I next proceeded to test for the presence of some form of mucilage, such as has been described by MANGIN (27) and WISSELINGH (28). With benzoazurin, Congo red, methyl green, corallin soda, aniline blue, I could detect no trace of colour in the matrix, but with concentrated Delafield's hæmatoxylin and with Bismarck brown a very definite bounding layer was seen, stained like the cell walls. These results are, however, quite insufficient to lead to any conclusion of a positive nature, especially as WISSELINGH (28) has shown that MANGIN's division of mucilages into cellulose, pectose, and callose mucilages no longer holds good. WISSELINGH has also proved in the cell walls of many fungi the existence of chitin strictly comparable to that which occurs in animal tissues. The method he adopts for its detection is as follows: Sections of alcoholic material are heated in concentrated potash to 160° C. for two hours. After cooling they are washed into 90 per cent. alcohol and then stained with iodine and sulphuric acid. If chitin be present a beautiful pink stain is given to the hyphæ, while the cells of the host take on the usual blue colour of cellulose. In this way WISSELINGH has shown chitin to be present in Myxomycetes, Phycomycetes, and numbers of other fungi, including some members of the Ustilagineæ, but he did not find it in any of the Bacteria which he investigated.

When applied to sections of Leguminous nodules this method gave no trace of pink colour ; we may, therefore, probably conclude that chitin does not enter into the composition of the matrix of these tubes—a fact which supplies additional evidence against the theory that the organism should be classed with the Ustilagineæ.

All I am able to offer concerning the matrix enclosing these rodlets is, therefore, of a negative character—that it contains nothing of the nature of cellulose or chitin, nor probably of mucilage.

The staining of the tube in the root-hair I found to be a very difficult matter, owing to the collapse of the hairs when the roots are transferred from one liquid to another, but I succeeded nevertheless in obtaining with strong hæmatoxylin and with aniline blue results which showed that here the tube consists of a chain of rodlets exactly like those present in the tubes within the tubercle cells (fig. 12).

The study of the infection tube at different stages down the hair led me to confirm FRANK's account of its open growing end. In the fresh condition, a rosette of refringent granules is generally to be noted at this end, such as suggests the exudation of some substance of the nature of a ferment by the contained organism as it advances down the hair, in a manner such as MARSHALL WARD (30) has described in the hyphæ of Botrytis.

If FRANK's view were correct, that the tube consisted of the plasma of the hair together with that of the invading organism, it seemed probable that the limits of the infection tube should correspond with the primordial utricle. To test this question I treated the hairs with different strengths of osmotically active substances. Eventually,

with a 5 per cent. solution of potassium nitrate, I obtained plasmolysis in the case of hairs containing infection tubes. As shown in fig. 13, this process demonstrated the truly parasitic nature of the tube, since beyond it was seen the ordinary protoplasmic contents of the hair. The tube, therefore, is actually formed by the parasite as it grows down the hair, and does not arise from the plasma of the host plant.

With regard to the reported absence of such tubes in *Lupinus* and *Phaseolus*, it is not improbable that this should be a physiological adaption of the organism to these particular hosts, rather than a reaction of the host to the parasite.

It is of interest to note that on very many roots on which I have grown tubercles, by direct inoculation of the seeds (experiments, which I shall describe later) I have found two, three, or more root-hairs with infection tubes passing down into one and the same tubercle; whilst, on the contrary, in plants which have grown in ordinary garden soil, one never finds more than one such infection tube entering each tubercle, and towards this one in all instances the axis of the tubercle is directed. Occasionally too, I have observed two tubes within the same root-hair; this, however, was a rare occurrence.

In the tubercles of all the species which I have examined the characters of the Bacteroids are the same. They consist of small straight or X and Y-shaped rodlets, which escape from cut cells with the well-known Brownian movement. They stain very readily with iodine, chlor-zinc iodine, hæmatoxylin, fuchsine, methylene blue, Bismarck brown, gold chloride, aniline blue, &c., hence the difficulty in obtaining a differentiating stain for the filaments only. At the close of the vegetative period, after flowers and fruit have been produced, the older tubercles consist of mere empty sac-like bodies, which contain no bacteroids, but simply a few straight rodlets and proteid bodies. This supports the theory that the Bacteroids have been absorbed by the plant, along with any nitrogen contained in them.

Summing up the characters thus far determined of this remarkable organism, it seems to be beyond doubt that the structure of the filaments—strands of rodlets, lying side by side in a homogeneous matrix—combined with the open free end of the infection tube and the absence of cellulose or chitin in the matrix, decide conclusively against its being one of the higher fungi. These characters indeed remind us at once of PRAZMOWSKI's view that the organism is a filamentous zoogloea form of a true Schizomycete, and also of the strange plants grouped together by THAXTER (15) under the name Myxobacteriaceæ.

Before, however, any final decision upon its systematic position can be arrived at, several other points require to be determined. We must know the complete life history of the rodlets contained in the tubes—whether their growth is apical or intercalary and how they become converted into " bacteroids "; whether the X, V and Y-shape forms arise by branching or by fusion. The determination of their mode of growth and multiplication is, of course, of special importance, since in this we have a crucial test of their Bacterial nature. As far as I am aware the only bodies met

with among plants, which strictly resemble the Bacteroids are the spores of *Proto-myces macrosporus*, formed by the conjugation of simple rod-like bodies. This suggests that the Bacteroids might be formed in a similar manner—by union of two or more individuals. The only other method which seems possible is that the rodlets should branch, thus giving rise to the well-known X, V, and Y-shapes. Branching in bacteria-like forms ("false-bacteria") has already been described by STUTZER and others in certain nitrifying organisms, and by MARSHALL WARD (31) in an organism which he obtained from the Thames. It is not therefore impossible that branching should occur in these rodlets also.

Answers to these questions can only be obtained by cultivating the rodlets outside the plant, by securing pure cultures of them, and by isolating them in drop cultures for continuous microscopic examination.

I have already drawn attention to the work which has been done in this direction, but until now, I believe no one has attempted the growth of these minute bodies in drop cultures kept under observation under high powers. In my experiments I employed a medium made up according to a formula given by BEYERINCK, viz., extract of pea-stems, gelatine 10 per cent. and asparagin $\frac{1}{4}$ per cent. On this material I succeeded in growing the "nitragin," supplied for agriculture, as well as organisms obtained directly from the tubercles.

In order to secure pure culture of these latter, I chose as large tubercles as possible, washed them carefully with $HgCl_2$ and alcohol, then with distilled water, and then cut them across quickly with a sharp razor previously heated in the flame. I then pierced the cut surface with a sterile platinum needle, with which streak cultures were made on slanting tubes of the gelatine medium.

From such cultures I next prepared one or more series of plates, until unmixed cultures were obtained. From these I infected a series of slanting tubes for future use. By this means I succeeded in growing the organism from *Vicia sativa, Pisum sativum*, and *Phaseolus multiflorus*.

If slanting tubes of gelatine, such as have been described be infected with "nitragin," good streaks are obtained in about two days. A microscopic examination of nitragin, or these sub-cultures from it, shows that the material consists of immense numbers of very minute bodies, scarcely longer than broad, all non-motile and similar in size and shape. No trace is found of the variety of shapes exhibited by the "Bacteroids."

In hanging drops, colonies are produced in the medium (best diluted to $2\frac{1}{2}$ per cent. gelatine) in about two days. By keeping such cultures under constant observation, I have succeeded in following the multiplication of the minute rodlets, both from nitragin and from cultures obtained direct from the tubercles.

At first the rodlet becomes slightly crescent-shaped, and by degrees a distinct con-striction is recognisable (fig. 16). The two portions later separate from one another for a short time before complete separation, a clear halo being visible round the two. This division into approximately equal halves has been followed in several individuals in

drop cultures of a gelatine medium and in simple pea-extract. The time required for the process is from two to four hours.

I had, thus far, failed to obtain any trace of the bacteroid forms in cultures on gelatine media, and I therefore determined to try the effect of a liquid medium consisting of a carefully prepared extract of pea-stems, which had been sterilised on four successive days. This material was infected with the organisms and kept perfectly still and protected from light. In five or six days a film is seen upon the surface, the rest of the liquid remains quite clear. An examination of drops from tubes treated in this way invariably showed that they contained numbers of straight rodlets, and mixed with these a considerable proportion of bodies with the well-known X and Y forms of Bacteroids. By dilution with freshly-sterilised pea-extract I have isolated organisms, from these cultures, in hanging drops, with a view to observing the actual formation of Bacteroids. Thus far, however, I do not feel prepared to offer any definite conclusions upon this question.

Consequently, though their multiplication by division lends support to the view that these organisms are members of the Schizomycetes, I prefer to withhold my opinion on this subject till further observations upon the last stage in their life-history have been made.

It is of some interest to mention that in no case have I been able to notice the growth of a " Bacteroid " under the microscope, whether in a solid or a liquid medium. Possibly the explanation of this is to be found in the view held by FISCHER (34), that the change from Bacteria to Bacteroids is a sign of death, and that in this form they are absorbed by the Leguminous plant.

As regards the size of the rodlets, my measurements give a mean of $\cdot99 \times 3\cdot3\,\mu$.

An interesting property of these organisms, at once obvious from any growth outside the plant, is that they are aërobic. It is difficult to realise by what means they are supplied with the necessary free oxygen within the cells of the tubercle. FRANK (10), it is true, has described the existence of air passages round each of the cells containing Bacteroids, but this has been again emphatically denied by SCHNEIDER (13). Personally, I have failed to observe any such structure; the cells, on the contrary, are always very compactly arranged, leaving no intercellular spaces. Both FRANK and SCHNEIDER agree, however, upon the occurrence of lenticels in the outer layers of the tubercles.

One other attempt I have made, up to the present, to cultivate this strange organism. Since it will grow upon dead organic media, it seemed probable that it would grow upon dead roots of some or any Leguminous plant. Accordingly I allowed pea seeds to germinate between layers of cotton wool, until the radicles were about one inch long. These I then dropped with sterile forceps into tubes containing well wetted plugs of cotton wool, the tubes having of course been very thoroughly sterilised beforehand. In these, the seedlings were steamed in a water bath for ten to fifteen minutes, in order to kill the roots. After cooling, I infected

the roots with drops of water containing nitragin, and then kept the tubes in the dark. In about ten days a good growth was obvious along the radicles, and upon examination the organism present appeared to be the one for which I was seeking. I consequently tried to separate it in a pure state by means of plate cultures, but all my attempts failed owing to a rapid liquefying of the gelatine medium. I have repeated this experiment upon numbers of seeds, but have not yet succeeded in isolating by this means the tubercle organism. Special difficulties naturally arise in this case from the ease with which numbers of other common Bacteria appear upon the seed itself, and thence doubtless spread to the radicle. To these probably the liquefying of the gelatine is due.

At this stage an important question naturally arises, viz., from what does the invading organism derive its nourishment during its growth down the root hair and across the cortex? Judging from the nature of the media, upon which it will grow outside the plant, it seems probable that the main substances should be of the nature of carbohydrates and proteids. By the use of methods such as HOFFMEISTER (29) has recently described, the presence or absence of sugars at least ought to be capable of demonstration.

In the next section of this paper my work is considered in its relation to the more practical side of the question, namely, the importance of the formation of tubercles to the well-being of the Leguminous plant, and the value to the agriculturist of a means whereby their formation can be procured with certainty, or if advisable, in increased numbers.

Some two or three years ago, Drs. NOBBE and HILTNER procured a patent for a germ-fertilizer, or "nitragin," as they have unhappily named it, for use in the cultivation of Leguminous crops.

This material, as supplied to farmers, consists of cultures on solid media of creamish-white streaks or colonies, which more or less cover the sloping surface of the gelatine, which is contained within an ordinary flattened bottle, securely closed by a cork plugged with cotton wool. The directions for use in agricultural work are briefly as follows :—By warming very slightly, melt the contents of the bottle and mix up with water, then either rub up the seeds in this before sowing, or pour the whole over the soil in which the seeds are to be planted. These directions being intended for ordinary agricultural conditions are necessarily very rough, it is consequently difficult to draw any trustworthy conclusions as to the value of nitragin from results based upon such methods, since the conditions of environment of the crops are so extremely complicated.

Consequently in order to secure more accurate conditions for experiment, I was obliged to diverge somewhat from the directions given. The plan I adopted was to remove from the surface of the gelatine some of the nitragin with a sterile platinum needle. This material I rubbed up in a small quantity of sterile distilled water, taking care that all the utensils employed were thoroughly sterile. For the purposes

of inoculation I employed this solution according to the two methods given on the labels.

For the growth of experimental plants I used small ordinary flower-pots, plugged with cotton wool or asbestos, and filled with pure sifted silver sand. The pots thus prepared, were heated for one hour in an oven at 120° C., and afterwards cooled. To each pot I then added 15 to 20 cub. centims. of a sterile solution of nutrient salts, and again subjected the pots to heat in a steam bath for one hour.

The solution of nutrient salts was made up according to SACHS' formula, except that KCl was added instead of KNO_3 with a view to rendering the conditions for infection as favourable as possible by limiting the supply of nitrogenous food to the nitrogen of the air.

Being anxious to avoid, if possible, the application of corrosive sublimate to the seeds, I made no attempt to sterilise them before sowing. My subsequent results with control experiments showed that this was quite justifiable. Provided the culture medium be well sterilised and only sterile water be used during growth, plants free from infection can be obtained from seeds which have undergone no special process of cleansing whatever. (These results, of course, confirm ZINSSER's conclusion that the Leguminosæ are not hereditarily infected with the tubercle organism.) After sowing, the pots were kept cool in a greenhouse, and protected from dust by bell-jars, which were raised occasionally to prevent too great accumulation of carbon dioxide. Throughout the experiments the plants were supplied with sterile distilled water only.

Obviously the first question to be decided was : Does this nitragin actually contain the organism in question ? a matter which could only be settled by its power to produce tubercles upon plants from seeds which had been sown in sterile media. For this purpose I prepared a series of pots, some containing seeds infected by nitragin, rubbed up in water, others, seeds without inoculation to serve as control plants. As the accompanying table shows, I have used seeds of various genera, inoculated with nitragin supplied for different genera or species. By these means I combined the process of testing the genuineness of nitragin and also the possibility of producing infection by the use of the organism proper for some other species or genus.

Before giving the details of my results in a tabular form, I may say in general that they were in all cases positive. The nitragin supplying the organisms from *Pisum* and *Vicia* was apparently identical in action, and the latter, when applied to seeds of *Lathyrus aphaca*, produced a considerable increase in the number of positive results as compared with plants which had not been thus treated.

A similar increase resulted from the use of the nitragin supplied for *Onobrychis* and *Lupinus* upon seeds of *Vicia hirsuta*.

The occurrence, in this series (*e.g.*, last column of series i.), of tubercles upon plants, cultivated as controls in soils which had been heated, illustrates a fact pointed

out by RICHTER (41) in 1896, viz., the extreme difficulty of keeping soil or sand sterile for many weeks in succession. Indeed, it has been often remarked that the organisms concerned in the formation of Leguminous nodules seem to be as ubiquitous, and consequently as difficult to remove as those of putrefaction and fermentation.

So far as I have tested the value of the inoculating material for *Lupinus* upon the Lupin seed, the results lead one to infer that it is comparatively small. This may, however, depend largely upon the composition of the culture medium, and I hope in future experiments to study the particular conditions affecting this genus more especially, and also the comparative value of the organism from plants of a given tribe when supplied to plants of other tribes of the order.

TABLE I.

Species planted.	"Nitragin" used for inoculation.	Sterilised medium.	Age of plants.	Number of seeds sown.	Percentage of plants showing infection.	Number of controls.	Percentage of infection without inoculation.
i. *Vicia hirsuta* . „ „	*Onobrychis sativa.* *Lupinus albus.* .	Soil Soil	1–4 months 1–4 months	70 38	per cent. 89 95	} 18	per cent. 67
ii. *Lupinus albus* . „ „	*Lupinus albus.* . „ „	Soil Ordinary unsterilised soil	5 months 5 months	23 20	4 13	} 15	0
iii. *Pisum sativum.*	*Vicia sativa* . .	Sand	18 days	27	85	26	0
iv. *Lathyrus aphaca*	*Vicia villosa* . .	Sand	1 month	35	50	15	20
v. *Vicia hirsuta* .	*Pisum sativum* .	Sand	6 weeks	20	100	15	0
vi. *Pisum sativum*	*Pisum sativum* .	Sand	1 month	24	62	18	0

In all these experiments the nitragin was applied directly to the seeds before sowing.

Series vi. of this set of experiments gave a lower percentage of positive results than was expected from former trials. This, I believe, may be mainly attributed to the fact that the plants were too much forced during the early stages of their growth, owing to the somewhat high temperatures reached during that time. As a result the plants wilted suddenly when about a fortnight old, and did not recover themselves again at all satisfactorily. I should mention, too, that with two exceptions, there were no tubercles yet formed on the roots. Infection had not got beyond the stage

of tubes within the root hairs.   I should also mention that on July 23rd I noticed on every pot groups of a whitish fungus, growing on the outside of the flower-pots and on the surface of the sand.   The plants themselves were in no case attacked.   This quickly changed to a beautiful coral pink, and on examination was found to consist of the fructifications of *Peziza confluens*, with ordinary asci and ascospores.   (It appeared also upon the pots which form the series given in Table V.)   This recalls to us the results which FRANK has described in a paper upon the effects of sterilisation of culture soils.   He found that *Pyronema Marianum* (*Peziza confluens*) grew more abundantly on sterilised than on unsterilised soils.

When it was first attempted to obtain inoculation by pouring over the culture soils water containing nitragin (*i.e.*, the second method of application given by Drs. NOBBE and HILTNER), the results obtained led to the conclusion that this method was less certain than that of applying the nitragin to the seeds themselves.   Subsequent experiments however showed this to be an erroneous conclusion.   For, side by side with the plants mentioned in Table I., series vi., were grown twelve plants of *Pisum sativum* in sterilised sand, to which water containing nitragin was then supplied. These seeds were sown on July 13th, and examined on August 5th and 8th.   Eight of the plants possessed young tubercles on the main roots, in addition to several infection tubes, the remaining four showed infection tubes only.   From this result we may conclude that infection in sand, mixed with nitragin, is both possible and certain.

From either of these methods it is, of course, impossible to learn anything definite of the conditions affecting the entrance into and the growth of the organism within the plants, but since direct inoculation of the seeds is possible, one is naturally led to the conclusion that it should be also feasible to infect the roots directly under conditions which could be observed and regulated.   ZINSSER (18) has already attempted this infection, by injecting the organisms into the tissues and by stroking the root-hairs with needles dipped into the inoculating material.   His results were in both cases negative.

Since, with the exception of *Lupinus*, and possibly *Phaseolus* (if we may accept FRANK's explanation of infection in these genera), infection always occurs in nature by the root-hairs, and never through the cells of the piliferous layer, it seems to be abnormal to use injection as a means of inoculation—such a method I have consequently rejected, and have confined myself to direct infection by external application of the organisms.   In my first attempts I chose *Vicia hirsuta* as my experimental plant, but partly owing to the small size of the plant, and partly to the difficulty which I experienced in procuring germination during the winter months, in my later experiments I used almost exclusively seeds of *Pisum sativum.*   This is in many respects a very convenient species, its seeds germinate readily, and after germination the seedlings grow rapidly and produce almost without exception very healthy young plants.

For this series of experiments I grew seedlings in sterile sand (under conditions similar to those for the plants grown in my other experiments) until the roots were about $1\frac{1}{2}$ to 2 inches in length, by which time a good crop of root-hairs was produced.

For the subsequent growth of the seedlings, I prepared a series of tubes in the following manner : I took some ordinary test-tubes about 1 inch in diameter, and lined them with clean filter-paper, and above and below inserted plugs of cotton-wool. To serve as covers for these tubes I prepared the same number of larger ones, which I merely plugged with wool at each end. The whole series were then heated in dry air for one hour. After this preliminary sterilisation I thoroughly saturated the lower plug and filter-paper linings of the smaller tubes with sterile water, or in some cases with a sterile solution of nutrient salts (SACHS' solution). The inner plug of the larger tubes I saturated with pure water. All the tubes were then steamed for one hour and allowed to cool (fig. 14).

After carefully removing the seedlings from the sand I infected the roots in one of the following ways :—

    i. By allowing a drop of the water containing nitragin (prepared as before) to trickle from a sterile needle, down the root-hairs.

    ii. By dipping the roots entirely into the solution.

After infection the seedlings were fixed into the smaller tubes by means of the upper cotton-wool plug, and the aërial organs covered by the large tubes. These were placed in boxes with pierced lids, in order to keep the roots darkened, and the boxes kept in a window with a good light.

Reference to the figure of one of these tubes will show that this method of treatment was quite harmless to the plants. From quite young seedlings they grew to plants with several inches of stem, and especially vigorous root systems. Nevertheless, with only one exception, all my results were negative, though each root was carefully examined for infection tubes in the hairs.

Since the roots, as was expected, invariably adhered closely to the filter-paper, I next tried to secure infection by pouring the nitragin solution down the sides of the tubes, thus obviating any damage to the delicate root-hairs, which was almost inevitable, however carefully the roots be touched, in either of the other methods. This series, however, again failed to show a single case of infection.

The details of my experiments are shown in the following table :—

TABLE II.

Methods of infection
$\begin{cases} a. & \text{Drop of solution, allowed to run down the root.} \\ b. & \text{Root dipped in the solution.} \\ c. & \text{Roots directed upon filter-paper, saturated with the} \\ & \quad \text{solution.} \end{cases}$

Species sown.	" Nitragin " used.	Method employed.	Date of—			No. of plants inoculated.	No. of positive results.
			Sowing.	Inocula-tion.	Exami-nation.		
i. *Vicia hirsuta* .	*Vicia sativa* . .	*a*	Jan. 19	Feb. 4	Feb. 18	4	1
ii. „ „ .	„ „ . .	*a*	„	„	„	3	0
iii. „ „ .	„ „ . .	*b*	„	Feb. 12	Feb. 26	1	0
iv. *Pisum sativum* .	*Vicia sativa* . .	*a*	Jan. 28	Feb. 8	Mar. 12	8	0
v. „ „ .	„ „ . .	*b*	Feb. 9	Feb. 16	Mar. 1	3	0
vi. *Pisum sativum* .	*Pisum sativum* .	*c*	Feb. 18	Feb. 26	Mar. 10 and Apr. 15	18	0

Such a remarkable series of negative results suggested either (as ZINSSER remarks) that, in order to become capable of producing infection, the organism must pass through the soil, or that infection is impossible after the root has grown beyond a certain stage. In order to test these points, I started another series of experiments upon seeds, which I divided into three sets as follows :—

a. I infected some seeds, exactly as described above, for ordinary sowing, and fixed them in tubes prepared as before, and left them to germinate.

b. I caused some seeds to germinate between layers of moist cotton-wool until the radicles were about half-an-inch long. I then infected the radicles with a drop of nitragin in water, and fixed the seedlings in tubes as before.

c. I infected some seeds as in *a* and left them to germinate as in *b*, then, after germination had begun, transferred them to tubes.

The details and results of this series are shown in—

TABLE III.

Species sown.	"Nitragin" used.	Method employed.	Date of—		No. of seeds sown.	No. of infections.
			Sowing.	Examination.		
1. *Pisum sativum*	*Vicia sativa* . .	*a*	April 21	May 11	8 (only 5 of which ger-minated)	4
2. „ „	*Pisum sativum* .	*a*	July 12	Aug. 9 and 10	4	2
3. „ „	*Vicia sativa* . .	*b*	April 20	May 19	4	2
4. „ „	*Pisum sativum* .	*b*	July 12	Aug. 5 and 9	8	8
5. „ „	*Vicia sativa* . .	*c*	April 21 transferred April 26	May 16	4	4

With three exceptions the seeds fixed in the tubes germinated quite satisfactorily, also, in the majority of cases, infection, when successful, had reached the stage of infection tubes, grown well down the hairs, and even into the root; in some instances, however, it was at a less advanced stage, but that it had occurred was obvious from the inflated and twisted ends of root-hairs, which are so constant a sign of successful attack by the nodule organism.

In group 4 of this series I noticed, on a very small piece of a lateral root from one of the plants, no less than 27 hairs, side by side, with well-grown infection tubes within them. This observation may serve to show how successful the attacks of this organism may be, provided suitable conditions can be arrived at.

It is at once clear from the above results that the success or failure of infection does not depend upon the passage of the organism through the soil, but evidently it is capable of growth upon the testa of the seed, and thence, by some as yet unex-plained means, it reaches the protruding radicle and attaches itself to and penetrates the root-hairs.

In addition to the series described in Table III., during November and December, some pea plants were grown as follows : the seeds were germinated in tubes, and after the radicles had grown to about half-an-inch in length, they were inoculated with nitragin. The young seedlings were then planted in sterilised sand. In all cases infection resulted within 12 days of inoculation. These results, of course, confirm those obtained by inoculation of the young radicle in the previous group of ex-periments.

From the positive results given by inoculation of the radicle shortly after germina-tion, we know that when the radicle is still quite young, and as yet has formed no root-hairs, direct infection from without is perfectly easy and certain. This at once suggests that the organism can only penetrate the wall of the hair, when it is in a merely incipient stage. That such is not the whole truth at any rate, is suggested

by certain observations which I made while studying the nature of the root-hair tube. In many root-hairs which had already reached their full size, tubes were present, which had but just begun to grow down the hair, or had not yet penetrated through its whole length. Similarly, in other fully formed hairs, infection had only just occurred, and the infection spot and inflated tip alone were evidences of the fact. On the contrary, I have also noted several very small root-hairs, scarcely larger than the cell from which they arose, penetrated by infection tubes which had already reached and entered the outer layer of the root-cells.

We are evidently confronted here by a difficult physiological problem as to the conditions which regulate the entrance of these organisms. Is it a case of chemio-taxis, depending upon certain variations in the composition of the excreta from the root-hairs; or does it result from the production by the invading organism itself of some form of ferment capable of dissolving the cell-wall; or again, is it a question of some special state of external conditions, which react either upon the organism or upon the roots, such as the chemical composition of the air surrounding the root-hairs, its degree of humidity, temperature, and so forth? Another possible cause of the negative results given in Table II. may have been the shock given to the roots in transferring them from the soil to the tubes. Some of these suggested points I have endeavoured to test in further experiments, others I hope to investigate in the future.

I first directed my attention to the effect of the unavoidable accumulation of carbon dioxide around the roots, when confined within tubes. By such a method of culture complete control over the plants had been obtained; consequently, in order not to lose this distinct advantage, my aim was to devise some form of tube, in which carbon dioxide could be absorbed, and thus be prevented from accumulating round the roots.

For this purpose I used tubes with a bulb below, separated from the main tube by a constriction (see fig. 15). These were lined and plugged above as before, and covering tubes prepared also. (These tubes were also wider and longer than those employed in the former series of experiments.) The tubes were then heated at 120° C. for one hour. I then removed the linings and plugs and passed down into the bulb a small stick of potash by means of a sterile glass rod with a spoon-shaped end. I then pushed down a sterile plug to close the constriction, replaced the lining of filter-paper, and saturated the whole with sterile water, before inserting the upper plug. The larger covering tubes were soaked with sterile water as before. An obvious disadvantage in this method was the impossibility of steaming the tubes after manipulation, but as all instruments employed were carefully sterilised beforehand, very little risk of infection was run. Side by side with seedlings which, after direct infection with drops of nitragin were placed in these tubes containing potash, I arranged a series, treated in exactly the same manner, except that no potash was added.

In all cases the plants thrived equally well, but, as Table IV. shows, again only one

positive result was obtained, and this occurred upon a seedling fixed in a tube containing water only, no potash. We may therefore conclude that the accumulation of carbon dioxide round the hairs is not the explanation of the failure to obtain direct infection in roots which have already produced abundant root-hairs.

TABLE IV.

Species planted.	"Nitragin" used.	Date of—			No. of plants inoculated.	No. of positive results.
		Sowing.	Infection.	Examination.		
1. *Pisum sativum* .	*Pisum sativum.* .	April 29	May 9	{ May 22 to June 6	{ With potash, 12 { Water only, 12	0 1
2. *Vicia hirsuta* .	„ „ . .	May 3	„ 14	{ June 11 to July 7	{ With potash, 12 { Water only, 10	0 0

In order to test the effects produced by changing the conditions under which the plants were growing, I arranged the following series of cultures, making use of *Pisum sativum* seeds and nitragin :—

i. Seeds were sown in pots of sterilised sand supplied with nutrient salts as before. After one week's growth they were removed from the sand and infected by drops of water containing nitragin, and fixed in tubes (as above) containing water only.

ii. Seeds were sown in pots as in i. After one week's growth the seedlings were removed and infected, and were then replaced in fresh pots of sterilised sand.

iii. Seeds were germinated in tubes, and, after the roots had grown to about 2 inches in length, they were inoculated, and the seedlings again fixed in tubes.

In each case the seedlings were allowed to grow for three weeks after inoculation. The results obtained are given in :—

TABLE V.

Name of species sown.	"Nitragin" used.	Date of—			No. of plants inoculated.	No. of positive results.
		Sowing.	Inoculation.	Examination.		
i. *Pisum sativum.* .	*Pisum sativum.* .	July 8	July 15	Aug. 8 and 10	12	4
ii. „ „ . .	„ „ . .	„ 8	„ 15	Aug. 8 and 10	12	10
iii. „ „ . .	„ „ . .	Nov. 1	Nov. 8 and 10	Nov. 29 and Dec. 3	6	4

In i. the four plants giving positive results showed good infection tubes on the lateral roots, not on the main roots.

In ii. and iii. infection tubes, when present, occurred either on the main or lateral roots, or on both.

This table, combined with Table III., series *c*, leads to the conclusion that a considerable change in the conditions of growth of the plant before and after inoculation, may have effect upon the result produced, and it seems probable that if these conditions are as far as possible unaltered, the shock inflicted upon the plant at the time of inoculation has no injurious effect upon either the host or the parasite.

Briefly summing up the results of my work on this subject, it leads to the following conclusions concerning the properties and value of nitragin :—

1. That nitragin does consist of the organism, which is involved in the formation of Leguminous nodules.
2. That it is possible to secure nodule formation when nitragin is applied to the plants in either of the ways advised by its producers, viz. :—
    A. Direct application to the seeds.
    B. Mixing with the culture soils.
3. In order to obtain infection of the radicle it is not necessary that the organism should pass through the soil.
4. The time of the year has no effect upon the infecting power of the organism.
5. Direct infection of roots, which have already produced good crops of root-hairs, though not impossible, is very rarely successful when the plants are placed under conditions differing from those under which germination took place, but if these conditions are not changed, infection is successful in a much larger percentage of instances.
6. Direct infection of quite young radicles is tolerably certain of success.

    (These last two conclusions are in partial agreement with ZINSSER'S proposition, that provided other conditions be favourable, the age of the plant has no effect upon the results of infection.)
7. The infection of plants of one genus by organisms proper to some other genus is certainly possible within the tribe Vicieæ.

    (My opinion as regards other tribes of the order I wish to reserve for the present.)

In connection with the more general question of tubercular swellings on roots of any plants, it may be interesting to note that I have in the course of my work on the Leguminosæ, also examined tubercles upon roots of plants of other orders. In no case, however, have any been found which show characters in common with those of the Leguminosæ ; for example :

Tubercles upon the roots of *Clematis stans* and *Clerodendron fœtidum* were sent to

me from the Botanic Gardens, Cambridge. Both these forms were due to hypertrophy of the root cells, resulting from the presence of nematode worms.

Again, tubercles were pointed out to me as occurring upon various species of *Eucalyptus*. These, too, were quite distinct from Leguminosæ tubercles, and consisted of very hard tissues containing quantities of tannin. The cause of their formation I did not determine.

In concluding this paper, I wish to record my thanks to Professor MARSHALL WARD for allowing me to carry out this work in his laboratory, and for the constant advice and help which he has given me throughout.

EXPLANATION OF PLATE.

PLATE 1.

Fig. 1. Tubercle of *Vicia hirsuta*. Cells of bacteroid tissue, showing swellings and breaks on the filaments. Section treated with Eau de Javelle.

Fig. 2. *V. hirsuta*. Section stained with gold chloride and formic acid.

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Fig. 5. Clover. Section treated with Eau de Javelle.

Figs. 1–5 were drawn with ZEISS Hom. Imm. $\frac{1}{12}$.

Figs. 6, 7, 8. Stages in the growth of the infection tube down the root-hair. S = spot of infection. Figs. 6 and 7 with obj. $\frac{1}{6}$. Fig. 8 Hom. Imm. $\frac{1}{12}$.

Fig. 9. *Pisum sativum*. Long. section of tubercle stained with aniline blue and orseillin, showing rodlets within the filaments. Hom. Imm. $\frac{1}{12}$ × oc. 4.

Fig. 10. *Pisum sativum*. Long. section of tubercle stained with methyl violet and fuchsin, showing liberation of rodlets from filaments. Hom. Imm. $\frac{1}{12}$ × oc. 4.

Fig. 11. *Vicia hirsuta*. Trans. section of tubercle, showing filament, with rodlets piercing the cell wall. ZEISS Apo. 1·5 millims. N.A. 1·30 × oc. 8.

Fig. 12. *Pisum sativum*. Infection tube in root-hair. Hom. Imm. $\frac{1}{12}$ × oc. 4. S = grains of sand on tip of hair.

Fig. 13. *V. hirsuta*. Root hair with infection tube, after plasmolysis with 5 per cent. potassium nitrate. n = nucleus.

Fig. 14. Culture tube used in series recorded in Tables II. and III. Scale $\frac{1}{2}$. Tube lined with filter-paper soaked with Sachs' solution. This pea seedling was grown from seed infected with nitragin for *Vicia sativa*, and germinated in the tube. Sown April 21 ; drawn May 11. r = roots.

Fig. 15. Culture tube used for series recorded in Tables IV. and V. In the former case potash was added in the bulb to absorb the carbon dioxide. Scale $\frac{1}{2}$. Seedling of *Pisum sativum*. Sown April 29 ; drawn May 31. r = roots.
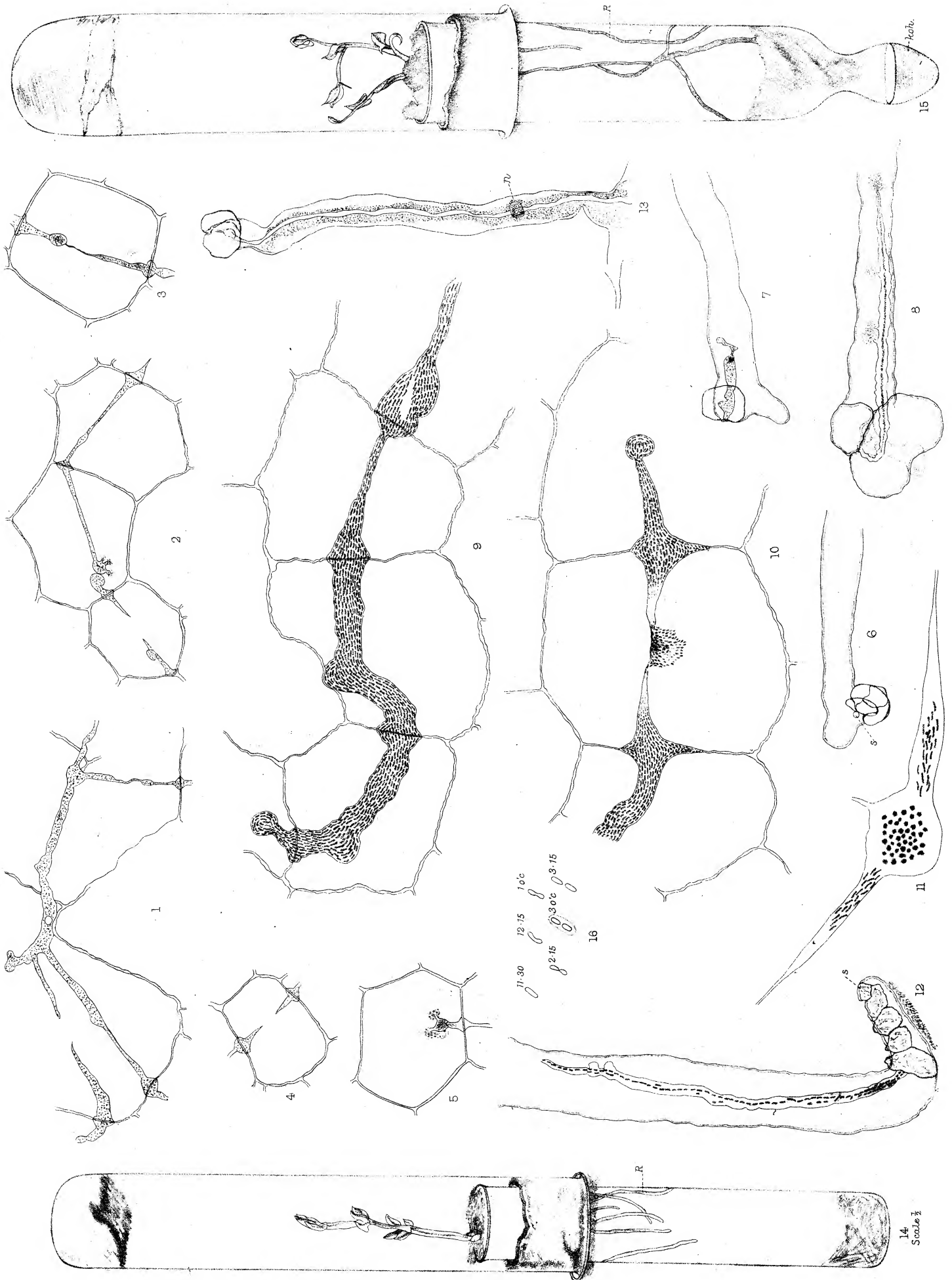
Fig. 16.   Stages in the division of rodlets, isolated from nitragin for *Pisum sativum*, and cultivated in a drop of pea-extract.   Temp. 17° C.   Obj. $\frac{1}{6}$.

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